Deletion analysis of the *Drosophila* Inscuteable protein reveals domains for cortical localization and asymmetric localization

Juergen A. Knoblich*†, Lily Y. Jan* and Yuh Nung Jan*

The *Drosophila* Inscuteable protein acts as a key regulator of asymmetric cell division during the development of the nervous system [1,2]. In neuroblasts, Inscuteable localizes into an apical cortical crescent during late interphase and most of mitosis. During mitosis, Inscuteable is required for the correct apical–basal orientation of the mitotic spindle and for the asymmetric segregation of the proteins Numb [3–5], Prospero [5–7] and Miranda [8,9] into the basal daughter cell. When Inscuteable is ectopically expressed in epidermal cells, which normally orient their mitotic spindle parallel to the embryo surface, these cells reorient their mitotic spindle and divide perpendicularly to the surface [1]. Like the Inscuteable protein, the *inscuteable* RNA is asymmetrically localized [10]. We show here that *inscuteable* RNA localization is not required for Inscuteable protein localization. We found that a central 364 amino acid domain – the Inscuteable asymmetry domain – was necessary and sufficient for Inscuteable localization and function. Within this domain, a separate 100 amino acid region was required for asymmetric localization along the cortex, whereas a 158 amino acid region directed localization to the cell cortex. The same 158 amino acid fragment could localize asymmetrically when coexpressed with the full-length protein, however, and could bind to Inscuteable *in vitro*, suggesting that this domain may be involved in the self-association of Inscuteable *in vivo*.

Address: *Departments of Physiology and Biochemistry, Howard Hughes Medical Institute, University of California, San Francisco, California 94143-0725, USA.

Present address: †Research Institute of Molecular Pathology (I.M.P.), A-1030 Vienna, Austria.

Correspondence: Juergen A. Knoblich
E-mail: knoblich@nt.imp.univie.ac.at

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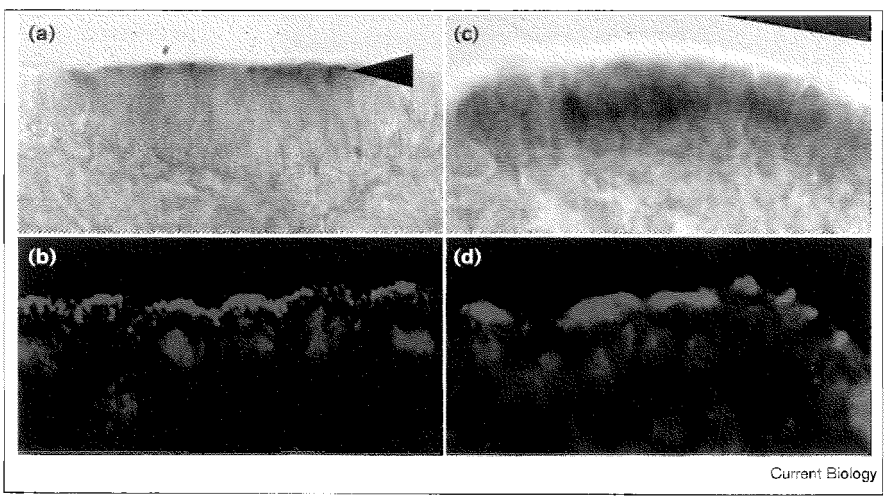
Results and discussion

In *Drosophila* neuroblasts [1,10,11] and in epithelial cells of the procephalic neurogenic region (PNR) (Figure 1a,b), both the *inscuteable* RNA and the Inscuteable protein are asymmetrically localized. To test whether asymmetric localization of the *inscuteable* RNA is required for Inscuteable protein localization, we expressed the *inscuteable* open reading frame, without the 5′-untranslated and 3′-untranslated regions of the gene, in epidermal cells (outside the PNR) of stage 10 *Drosophila* embryos. Even though the RNA produced from this transgene was homogeneously

Figure 1



Asymmetric localization of *inscuteable* RNA is not required for asymmetric localization of Inscuteable protein. (**a**) Localization of *inscuteable* transcripts (arrowhead) in the apical cell cortex of PNR cells in a stage 9 wild-type *Drosophila* embryo processed for *in situ* hybridization using a full-length *inscuteable* probe. (**b**) Localization of Inscuteable protein to the apical cell cortex of PNR cells in a wild-type stage 9 *Drosophila* embryo stained with an antibody against Inscuteable (shown in green) and for DNA (shown in red). (**c**) Transgenic *Drosophila* embryos expressing the Insc–Myc construct under the control of the *hairy* promoter using the UAS–GAL4 system [16]. The *insc–myc* RNA was visualized by *in situ* hybridization using an *inscuteable* full-length probe. Epithelial cells outside the PNR (which do not express endogenous *inscuteable*) are shown. (**d**) Embryos from (c) were stained with an anti-Inscuteable antibody (shown in green) and for DNA (shown in red).

distributed throughout the cell (Figure 1c), the Inscuteable protein was still asymmetrically localized to the apical cortex of epithelial cells (Figure 1d). Similar observations were made when the same transgene was expressed in neuroblasts (data not shown). Thus, asymmetric localization of *inscuteable* transcripts is not required for the asymmetric localization of the Inscuteable protein.

The Inscuteable protein has no overall homology to other known proteins, but contains a proline-rich region that matches the consensus for binding to Src homology 3 (SH3) domains and for binding to WW domains [12] and a carboxy-terminal motif that matches the consensus for binding to PDZ domains [13]. To identify the protein motifs responsible for asymmetric localization and function, we expressed seven Myc-epitope-tagged deletion mutants of Inscuteable in epidermal cells (outside the PNR) and tested them for asymmetric localization and their ability to cause reorientation of mitotic spindles in these cells (Figure 2a). Full-length Insc–Myc (Figure 2b), as well as deletions Insc-Δ1, Insc-Δ2, Insc-Δ6 and Insc-ΔC (see Supplementary material published with this paper on the internet) localized exclusively to the apical cell cortex and caused spindle reorientation. In contrast, Insc-Δ3 was homogeneously distributed in the cytoplasm and Insc-Δ4 was localized to the cell cortex with no signs of asymmetric localization (see Supplementary material). Both Insc-Δ3 and Insc-Δ4 had no effect on mitotic spindles. Insc-Δ5 was partially localized asymmetrically (Figure 2c) and caused spindle reorientation only in a subset of the cells that expressed the protein (Figure 2i). We conclude that the central region of the Inscuteable protein is required for asymmetric localization and spindle reorientation, but neither the proline-rich region nor the putative PDZ-binding domain are essential for these processes.

To test whether the central part of the Inscuteable protein is also sufficient for localization and function, we expressed amino acids 252–615 of the Inscuteable protein either fused to a Myc tag (Insc-cen–Myc) or fused to the β-galactosidase (β-gal) protein (Insc-cen–β-gal) in transgenic flies and determined their subcellular localization and effect on spindle orientation by immunofluorescence. Both fusion proteins were found exclusively at the apical cell cortex in wild-type epidermal cells (Figure 2d,e) and neuroblasts (data not shown), as well as in *inscuteable* mutant neuroblasts (Figure 3); these proteins caused reorientation of mitotic spindles in wild-type epidermal cells (Figure 2j,k). In contrast, amino acids 302–459 of Inscuteable (the region deleted in Insc-Δ3) fused to β-gal (Insc-03–β-gal) were localized to the cell cortex with no signs of asymmetric localization in epidermal cells (Figure 2f, but see below),

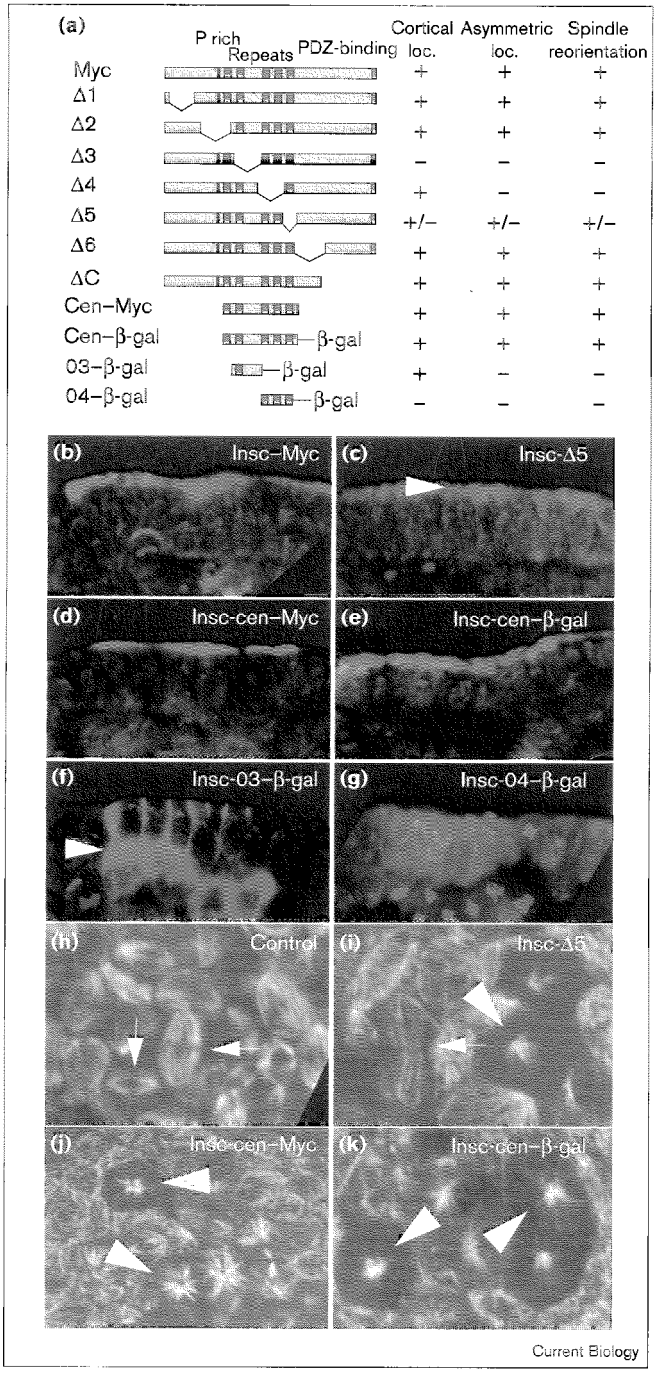
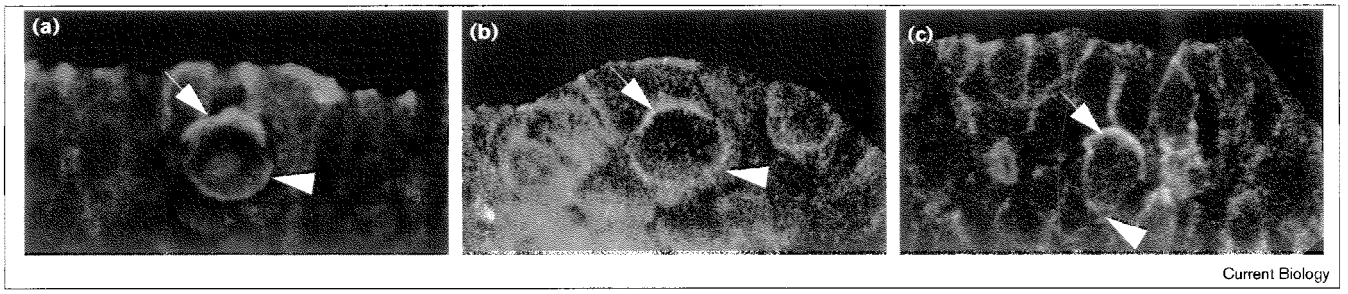


Figure 2

Analysis of Inscuteable deletion mutants. **(a)** Summary of subcellular localization and function of Inscuteable deletion and fusion constructs. All constructs lack the *inscuteable* 5′-untranslated and 3′-untranslated region and an SV40 polyadenylation signal. Red boxes, proline-rich region (P rich); green boxes, central repeats; blue boxes, putative PDZ-domain-binding site. **(b–g)** The indicated Inscuteable deletion and fusion constructs were expressed in epidermal cells (outside the PNR) using a *hairy–GAL4* line [16] and their subcellular localization was determined by immunofluorescence (Inscuteable proteins shown in green, DNA in red). **(h–k)** Mitotic spindles in epidermal cells expressing the indicated Inscuteable mutants were visualized by anti-β-tubulin staining. Epidermal cells from stage 10 embryos are shown. Arrows indicate cells with normal mitotic spindles, and arrowheads indicate cells with reoriented spindles.

Figure 3



Insc-cen–β-gal can rescue the Miranda, Prospero and Numb localization defects in *inscuteable* mutant embryos. Insc-cen–β-gal was expressed in *insc*^{P72} mutant embryos under the control of the *hsp70* promoter using the UAS–GAL4 system [16]. Embryos were costained for β-galactosidase (shown in green) and **(a)** Miranda, **(b)** Prospero or **(c)** Numb (shown in red). Embryos in (a) are also stained for DNA (shown in blue). In all metaphase neuroblasts ($n = 50$ in each case) that expressed detectable amounts of Insc-cen–β-gal, the Insc-cen–β-gal protein was localized to the apical cell cortex (arrow) and (a) Miranda, (b) Prospero and (c) Numb were localized in a basal cortical crescent (arrowhead).

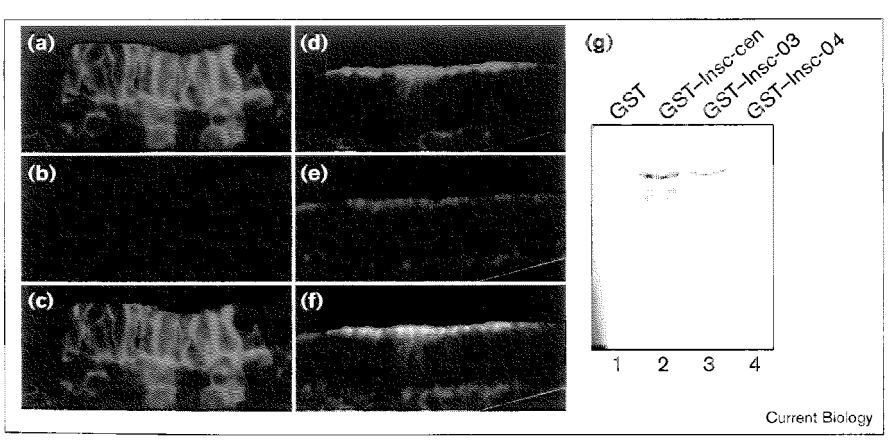
whereas amino acids 436–552 of Inscuteable (the region deleted in Insc-Δ4) fused to β-gal (Insc-04–β-gal) were found in the cytoplasm (Figure 2g). Neither Insc-03–β-gal nor Insc-04–β-gal had an effect on spindle orientation in epidermal cells (data not shown). We conclude that amino acids 252–615 of Inscuteable are sufficient for asymmetric localization and for inducing spindle reorientation in epidermal cells, whereas the cortical localization domain is located within amino acids 302–459.

In wild-type neuroblasts, the proteins Numb, Prospero and Miranda localize asymmetrically into crescents overlying the basal centrosome and segregate into the basal daughter cell [3,5–9]. In *inscuteable* mutant neuroblasts, all three proteins either fail to form crescents or form crescents at random positions of the cortex; these crescents are no longer correlated with the mitotic spindle [1]. To determine whether this function of Inscuteable is also mediated by the central domain, we expressed the Insc-cen–β-gal protein in *inscuteable* mutants. Despite expression from a ubiquitous promoter, many neuroblasts showed no detectable expression of the Insc-cen–β-gal protein and, in these cells, Miranda, Prospero and Numb localization were still defective (data not shown). We therefore analyzed 50 neuroblasts from *inscuteable* mutant embryos in which the Insc-cen–β-gal protein could be detected with an anti-β-galactosidase antibody (see Supplementary material). In all cases, the Insc-cen–β-gal fusion protein was localized to the apical cell cortex and the Miranda (Figure 3a), Prospero (Figure 3b) and Numb (Figure 3c) crescents formed at the opposite, basal cell cortex. Moreover, DNA staining revealed that the metaphase plate was oriented parallel to the embryo surface in all of these neuroblasts, indicating that Insc-cen–β-gal could also rescue the spindle orientation defect in *inscuteable* mutants (Figure 3a and data not shown).

Our results indicate that amino acids 252–615 of Inscuteable are sufficient for directing and orienting asymmetric cell divisions in *Drosophila* neuroblasts. We have therefore named this region the Inscuteable asymmetry domain.

Figure 4

Self-association of Inscuteable. **(a–f)** Insc-03–β-gal was expressed in epidermal cells using a *hairy*–GAL4 line [16]. Heat-shocked embryos that either (a–c) did not express or (d–f) did express a *hsp70*-promoter-driven full-length *inscuteable* transgene were stained with anti-β-galactosidase antibodies (green, to visualize Insc-03–β-gal, a,d) and anti-Inscuteable antibodies (red, to visualize full-length Inscuteable, b,e). The merged image of (a,b) is shown in (c) and of (d,e) in (f). **(g)** Co-immunoprecipitation analysis of *in vitro* translated [^{35}S]-labelled full-length Inscuteable protein precipitated with GST (lane 1), GST–Insc-cen (lane 2), GST–Insc-03 (lane 3) or GST–Insc-04 (lane 4). Precipitated proteins were separated by SDS–PAGE.



This domain contains five repeats of limited similarity [2] that are characterized by the core motif VRxL/I (in single-letter amino acid code where x represents any amino acid). Further experiments are required to directly test the functional significance of this motif. Amino acids 302–459 of Inscuteable localize to the cell cortex but fail to localize asymmetrically, whereas deletion of amino acids 436–535 affects asymmetric localization but not cortical localization. Thus, Inscuteable localization may be a two-step process involving cortical localization directed by amino acids 302–459 and asymmetric localization along the cell cortex directed by amino acids 436–535. Results suggestive of a similar two-step process have been obtained in a deletion analysis of the *Drosophila* Numb protein [14].

Insc-03–β-gal did not localize asymmetrically in epidermal cells (Figure 2f) but could localize asymmetrically when expressed in neuroblasts of wild-type embryos (arrowhead in Figure 2f). Inscuteable is expressed in neuroblasts but not in epidermal cells (Figure 4). When coexpressed with full-length Inscuteable, the Insc-03–β-gal protein was found exclusively at the apical cell cortex (Figure 4d–f). In contrast, in the absence of full-length Inscuteable, Insc-03–β-gal was not restricted to the apical cell cortex (Figure 4a–c), nor was Insc-03–β-gal asymmetrically localized in neuroblasts of *inscuteable* mutant embryos (data not shown). This indicates that this fusion protein can only localize asymmetrically in the presence of full-length Inscuteable. To test for a direct interaction between these proteins, we performed *in vitro* co-immunoprecipitation experiments. *In vitro* translated full-length Inscuteable protein could be precipitated by a fusion protein containing glutathione-S-transferase (GST) fused to amino acids 252–615 of Inscuteable (GST–Insc-cen) or by a GST–Insc-03 fusion protein (containing amino acids 302–459) bound to glutathione beads. In contrast, neither GST–Insc-04 (containing amino acids 436–552) nor GST alone bound to glutathione beads was able to precipitate Inscuteable. Taken together, these experiments suggest that the Insc-03 fragment binds directly to Inscuteable.

Materials and methods

Immunofluorescence and in situ *hybridization experiments*

Immunofluorescence experiments were performed as described before [3]. Primary antibodies were rabbit-anti-Numb [3], rabbit-anti-Inscuteable [1], rabbit-anti-Prospero [15], rabbit-anti-Miranda [8], rabbit-anti-β-gal (Promega), mouse-anti-β-tubulin (Amersham) and mouse-anti-Myc (9E10, Santa Cruz Biotechnologies). *In situ* hybridization experiments were performed using standard protocols. A 2.4 kb *Bam*HI fragment containing most of the *inscuteable* open reading frame was labelled to generate a digoxigenin-labelled probe.

Co-immunoprecipitation experiments

To express GST–Insc fusion proteins, a 2.4 kb *Bam*HI fragment containing most of the *inscuteable* coding region or PCR fragments containing amino acids 252–615 (GST–Insc-cen), 302–459 (GST–Insc-03) or 436–552 (GST–Insc-04) were cloned into pGEX4T-1 (Pharmacia). Fusion proteins and GST alone (empty vector) were expressed in the bacterial strain HB101 and were bound to glutathione beads (Pharmacia). Full-length *inscuteable* was translated *in vitro* using the TNT kit (Promega), bound to the beads for 30 min and washed 6 times with PBS containing 0.1% NP40. The beads were boiled in SDS sample buffer and proteins were separated by SDS–PAGE. Gels were dried and exposed to X-ray film overnight.

Supplementary material

Additional methodological detail and a figure showing the localization and function of Inscuteable deletion mutants is published with this paper on the internet.

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